

Binding of a spin-labeled phenylalanine analog to sickle hemoglobin: EPR and NMR studies

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We have synthesized a spin-label analog of phenylalanine as a competitive inhibitor probe of the sickle hemoglobin aggregation process. Sickle hemoglobin gelation measurements indicate that the spin-label phenylalanine analog is a potent inhibitor of deoxy sickle hemoglobin aggregation. We have also used spin label EPR and high-resolution proton NMR to study the interaction of the phenylalanine analog with hemoglobin, and find that the kinetic off-rate is comparable to, or slower than the hemoglobin rotational rate (i.e., $\geq 10^8 \text{ s}^{-1}$), and that at least one, and perhaps two significant localized interaction region(s) exist within a few angstroms of the β chain N- and C-termini. Correlation with other known structural information suggests that the observed interaction sites may be relevant to the mechanism for inhibition of sickle hemoglobin aggregation.

Hemoglobin Sickle EPR NMR Spin label Competitive binding

1. INTRODUCTION

Recent studies have shown the existence of a binding interaction between Hb and a variety of small molecules with hydrophobic moieties [1–8]. NMR studies have shown that several benzene derivatives exhibit weak, but specific binding to COHb, deoxy Hb and metHb [1,2]. Relaxation studies indicate that an aromatic binding site is within a few angstroms of the heme iron, but no detailed binding site has been proposed [1]. Several small molecules containing a variety of hydrophobic moieties also inhibit the aggregation or gelation

of deoxygenated HbS through noncovalent interactions [3–7]. The relative hydrophobicity of an agent appears to be the dominant characteristic in determining its effectiveness as an aggregation inhibitor [5]. These agents appear to act by a competitive binding mechanism in which the inhibitor binds to the HbS molecule at a crucial intermolecular contact site, and prevents the association of two HbS molecules at that site. Recent X-ray crystallographic studies of COHb and deoxy Hb co-crystallized with several aromatic aggregation inhibitors have suggested the existence of binding sites near $\alpha 14$ Trp, and in the internal cavity between the subunits [8]. To elucidate further the binding site for these small molecules under solution conditions, we have recently used conventional and spin label-induced NMR relaxation to study the interaction of phenylalanine with COHbS [9]. We found that Phe exhibits weak binding to Hb, and that a binding site is probably located within about $10 \pm 3 \text{ \AA}$ of the nitroxide free electron of hemoglobin that is spin-labeled at the $\beta 93$ Cys residues [9]. This is consistent with the

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Abbreviations: Hb, hemoglobin; COHb, carbonmonoxy liganded ferrous Hb; metHb, ferric Hb; HbA, normal adult Hb; HbS, sickle Hb; Tempone, 2,2,6,6-tetramethyl-4-oxopiperidone-1-oxyl; SL-Phe, *N*-(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carboxyl)-L-phenylalanine *t*-butyl ester; Phe, phenylalanine; Cys, cysteine; Bis-Tris, [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane

binding site being located within the lateral contact site that is complementary to the $\beta 6$ mutation site within the deoxy HbS polymer structure [3,10].

Here, we report spin label EPR and high-resolution NMR measurements on the binding of a spin label analog of Phe (SL-Phe) to COHbS. The EPR data indicate that SL-Phe exhibits moderate binding to Hb, with a binding residence time comparable to, or longer than the rotational correlation time of Hb. The NMR data suggest that there is at least one, and perhaps two significant interaction region(s) close to the $\beta 2$ His and $\beta 146$ residues on the Hb surface. Both of these residues are close to important HbS-HbS intermolecular contact sites [10], thus the interaction may be significant for the action of Phe and related molecules as HbS aggregation inhibitors.

2. MATERIALS AND METHODS

Sickle blood samples were obtained from homozygous donors, and Hb composition was checked by electrophoresis. Samples containing more than trace amounts (5%) of HbA or fetal Hb were discarded. Blood samples of HbA were obtained from the University of Illinois Hospital Blood Bank. Membrane-free HbA and HbS were prepared from the blood samples as in [11], and kept under CO atmosphere until use. The Hb solutions used in EPR studies were further purified by running through a Sephadex G-25 column with 0.15 M Na-PO₄ buffer at pH 7.2. The spin probe, Tempone, was purchased from Eastman, and SL-Phe was synthesized in our laboratory; details of the synthesis will be published elsewhere.

Measurements of the deoxy HbS gelation inhibitory effectiveness of Phe and SL-Phe were performed using the ultracentrifugation C_{sat} assay described in [12] at 20°C.

For EPR measurements, samples containing 0.1 mM Tempone or SL-Phe and COHb at a concentration of about 30 g/dl in 0.15 M Na-PO₄ buffer at pH 7.2 were prepared under N₂ atmosphere and loaded into 50- μ l capillary pipettes. Conventional first harmonic EPR spectra were measured at 20 mW microwave power, 100 G sweep width and 2 G modulation amplitude on a Varian E-4 spectrometer at 25°C.

Organic phosphate was removed from Hb samples as in [13]. All solutions for NMR studies

were treated with Chelex 100 (BioRad) to remove paramagnetic ions [9]. The Hb solutions were then exchanged 5 times with 0.1 M Bis-Tris buffer in D₂O, to suppress the intense H₂O proton resonance in the ¹H NMR spectrum. All glassware was soaked in alkaline EDTA solution overnight and washed with distilled, deionized water to remove paramagnetic ions. COHb solutions at a concentration of 10 g/dl, containing 0.5 mM Tempone or SL-Phe, were prepared under N₂ atmosphere and loaded into NMR tubes. The final pH of the samples was 7.4; values are given here as direct pH meter readings, without correcting for the deuterium isotope effect on the glass electrode, namely pD = pH + 0.4 [14]. The accuracy of each pH measurement was estimated to be ± 0.02 pH unit. ¹H NMR spectra were obtained by the rapid-scan cross-correlation technique [15] on a Nicolet 360 MHz spectrometer at a probe temperature of 21°C. The spectrometer was locked on the D₂O signal in each sample. The sweep width was 1800 Hz, with a sweep time of 5 s and a pre-delay time of 1 s.

3. RESULTS AND DISCUSSION

The ratios of deoxy HbS solubility in the presence of gelation inhibitor (SL-Phe or Phe) to the solubility in the absence of any inhibitors, C_1/C_0 , at various inhibitor concentrations are shown in fig.1. The slope of the ratio, C_1/C_0 , as a function of increasing inhibitor concentration,

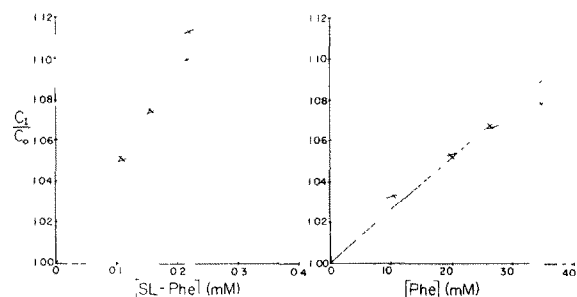


Fig.1. Deoxy HbS solubility ratio, C_1/C_0 , as a function of added SL-Phe or Phe. C_1 is the deoxy HbS solubility in the presence of the gelation inhibitor, while C_0 is the solubility in the absence of inhibitor. The ratio has been assumed to be directly proportional to inhibitor concentrations and to have a value of 1.0 in the absence of inhibitor. The slope is a measure of the effectiveness of the inhibitor.

can be taken as a measure of the inhibitor effectiveness. The data of fig.1 indicate that the spin-labeled Phe analog is about 190-times more potent than Phe as an inhibitor of deoxy HbS gelation. This increase in inhibitory effectiveness is probably largely the result of the enhanced hydrophobic character of the Phe analog.

EPR spectra of SL-Phe alone in solution and in the presence of COHbS, and of Tempone in the presence of COHbS are shown in fig.2. For SL-Phe in solution without any Hb, the spectrum is the simple 3-line pattern expected for a nitroxide tumbling freely in solution. For SL-Phe in the presence of COHbS, an additional spectral component characteristic of slow rotational tumbling ($\tau_r = 10^{-7}$ – 10^{-8} s) appears in the spectrum. The only change in solution condition is the addition of protein, thus the slow motion component must result from SL-Phe bound to Hb. A solution of Tempone plus Hb under the same conditions yields only the sharp 3-line fast motion spectrum, thus the binding of SL-Phe to Hb is also a consequence of the structure of the Phe analog, rather than of non-specific nitroxide binding to Hb. Taken together, these results indicate that SL-Phe ex-

hibits specific binding to Hb, and that the residence time for at least one binding site must be comparable to, or longer than the Hb rotational correlation time [τ_r (Hb) \approx 30 ns].

^1H NMR spectra of the aromatic resonances for COHbS alone in solution, in the presence of SL-Phe, and in the presence of Tempone are shown in fig.3. Resonances for the C2 and C4 protons of $\beta 2$ His, and for the C2 proton of $\beta 146$ His have been previously assigned [16], and are labeled in the top spectrum. Labeling of resonances follows the pattern given by [16]. Addition of 0.5 mM Tempone to the Hb sample produces perhaps a small amount of general line broadening, but no specific changes in the spectrum, suggesting that the small nitroxide exhibits no specific interaction with Hb – consistent with the EPR results discussed above. Upon adding 0.5 mM SL-Phe to the system, however, very substantial spectral changes take place. Resonances C, G, P and S are almost totally suppressed, and other resonances are moderately

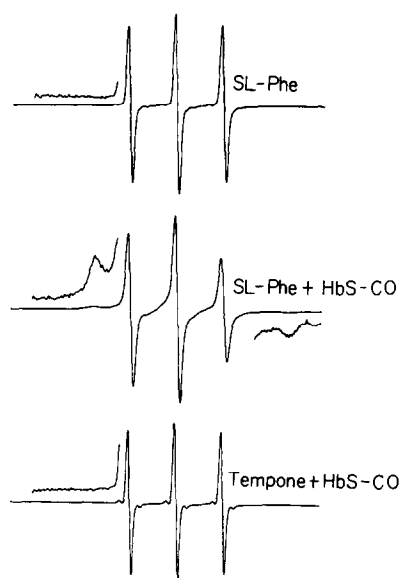


Fig.2. (Top) EPR spectrum of 0.1 mM SL-Phe in solution. (Center) EPR spectrum of 0.1 mM SL-Phe plus 30 g/dl COHbS in solution. (Bottom) EPR spectrum of 0.1 mM Tempone plus 30 g/dl COHbS in solution. Buffer conditions are as noted in the text.

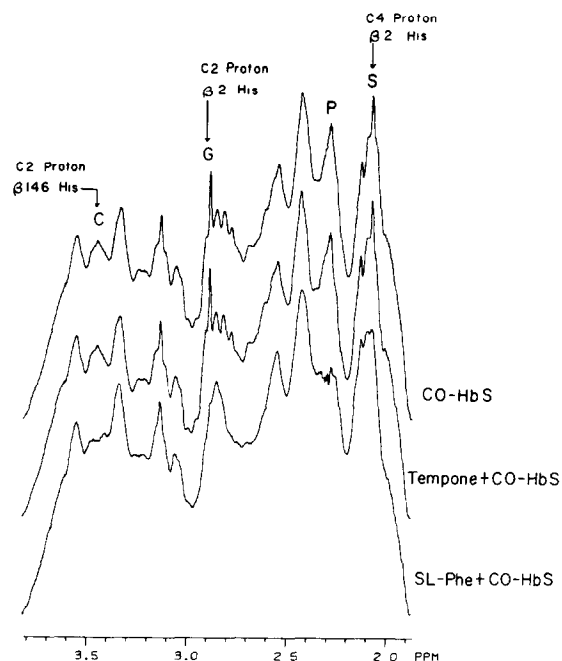


Fig.3. (Top) ^1H NMR spectrum of 10 g/dl COHbS in solution. Labeling and assignment of $\beta 2$ and $\beta 146$ resonances are taken from [15]. (Center) ^1H NMR spectrum of 10 g/dl COHbS plus 0.5 mM Tempone in solution. (Bottom) ^1H NMR spectrum of 10 g/dl COHbS plus 0.5 mM SL-Phe in solution. Buffer conditions are as noted in the text.

broadened. This is essentially the behavior to be expected if SL-Phe interacts with Hb at one or more localized regions. Resonances arising from protons located close to the interaction region(s) will experience strong paramagnetically induced relaxation from the fluctuating electron paramagnet of the nitroxide, and may be broadened enough to be no longer detectable [17]. Resonances arising from protons located farther from the interaction regions would be broadened much less due to the r^{-6} distance dependence of paramagnetically induced relaxation.

Resonances G and S arise from the C2 and C4 protons, respectively, of $\beta 2$ His, thus SL-Phe apparently interacts with the Hb surface at a location within a few angstroms of the β -chain N-terminus. Resonance C arises from the C2 proton of $\beta 146$ His, thus SL-Phe apparently also interacts with the Hb surface at a location within a few angstroms of the β -chain C-terminus.

Resonance P may also be from a His C4 proton, but has not been assigned to a specific residue. Its disappearance in the presence of SL-Phe suggests either that it is also from a residue located near the ' $\beta 2$ ' or ' $\beta 146$ ' regions, or that another interaction site exists.

The sickle hemoglobin mutation results in the substitution of valine for glutamic acid at the $\beta 6$ position. Thus, the SL-Phe binding site that is near $\beta 2$ His is probably also in close proximity to the mutation site, and may well play a role in the inhibition of HbS aggregation. Within the Hb molecule, the two β chains, β_1 and β_2 , are oriented 'antiparallel' to each other, with the β_1 N-terminus close to the β_2 C-terminus, and vice versa. The side chain imidazole rings of the β_{12} and β_{2146} His residues are located within about 10–12 Å of each other (as are those of the β_{146} and β_{22} His residues) [18]. Thus interaction of SL-Phe with Hb at a single local region that is close to both the $\beta 2$ and $\beta 146$ His residues could produce suppression of resonances C, G and S (and perhaps P). However, the C-terminal $\beta 146$ His residue is also located near the F helix region of the β chain, and is within about 10–15 Å of the $\beta 85$ Phe, $\beta 88$ Leu and $\beta 91$ Leu residues that we previously proposed as a binding site for small hydrophobic molecules [9]. The relatively long binding residence time suggested by the EPR spectra of fig.1 would probably be sufficient to induce suppression of the $\beta 146$ His

resonances. Thus another significant interaction region for SL-Phe may also exist. This region appears to be an important HbS-HbS intermolecular contact region [10], thus interaction at this location may also play a role in the inhibition of deoxy HbS gelation. Authors in [8] have also suggested that binding of aromatic gelation inhibitors may occur near $\alpha 14$ Trp, and at sites in the internal cavity between subunits, thus interaction at several sites may be possible.

In previous work we have shown that even fully carbon monoxide liganded HbS exhibits a limited aggregation under some conditions, with such aggregation presumably continuing upon deoxygenation to produce the commonly observed polymer structures [19,20]. The binding behavior observed here appears to be fully consistent with a competitive binding mechanism for inhibition of HbS gelation, however more detailed studies including liganded and deoxy forms of both HbA and HbS will be required to elucidate fully inhibitor binding sites for the liganded and unliganded states, and to determine those sites that are important under solution conditions.

In summary, we have synthesized a spin-labeled analog of phenylalanine that inhibits deoxy HbS aggregation about two orders of magnitude better than the parent amino acid. EPR measurements indicate that SL-Phe exhibits significant binding to COHbS, with a binding residue time that is long on the EPR time scale. NMR measurements suggest that one or two localized interaction regions are physically close to the $\beta 2$ His and $\beta 146$ His residues. Binding near the $\beta 2$ His should influence the $\beta 6$ mutation site, and inhibit HbS gelation; binding near the $\beta 146$ His residue is consistent with our previous suggestion of a hydrophobic binding site being located in the cleft between the β -chain F helix and the heme [9], and may also play a role in the inhibition of HbS gelation.

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tion (M.E.J.). The COHb crystal coordinates were obtained from the Brookhaven protein data bank.

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